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INCREASES IN MATURE BRAIN-DERIVED NEUROTROPHIC FACTOR PROTEIN IN THE FRONTAL CORTEX AND BASAL FOREBRAIN DURING CHRONIC SLEEP RESTRICTION IN RATS: POSSIBLE ROLE IN INITIATING ALLOSTATIC ADAPTATION

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Abstract—Chronic sleep restriction (CSR) has various negative consequences on cognitive performance and health. Using a rat model of CSR that uses alternating cycles of 3 h of sleep deprivation (using slowly rotating activity wheels) and 1 h of sleep opportunity continuously for 4 days ('3/1' protocol), we previously observed not only homeostatic but also allostatic (adaptive) sleep responses to CSR. In particular, non-rapid eye movement sleep (NREMS) electroencephalogram (EEG) delta power, an index of sleep intensity, increased initially and then declined gradually during CSR, with no rebound during a 2-day recovery period. To study underlying mechanisms of these allostatic responses, we examined the levels of brain-derived neurotrophic factor (BDNF), which is known to regulate NREMS EEG delta activity, during the same CSR protocol. Mature BDNF protein levels were measured in the frontal cortex and basal forebrain, two brain regions involved in sleep and EEG regulation, and the hippocampus, using Western blot analysis. Adult male Wistar rats were housed in motorized activity wheels, and underwent the 3/1 CSR protocol for 27 h, for 99 h, or for 99 h followed by 24 h of recovery. Additional rats were housed in either locked wheels (locked wheel controls [LWCs]) or unlocked wheels that rats could rotate freely (wheel-running controls [WRCs]). BDNF levels did not differ between WRC and LWC groups. BDNF levels were increased, compared to the control levels, in all three brain regions after 27 h, and were increased less strongly after 99 h, of CSR. After 24 h of recovery, BDNF levels were at the control levels. This time course of BDNF levels parallels the previously reported changes in NREMS delta power during the same CSR protocol. Changes in BDNF protein levels in the cortex and basal forebrain may be part of the molecular mechanisms underlying allostatic sleep responses to CSR.

Key words: sleep deprivation, Western blot, basal forebrain, frontal cortex, hippocampus, allostasis.

INTRODUCTION

Chronic sleep restriction (CSR), i.e., not obtaining adequate sleep over days, weeks, to even years, has become common in modern societies due to various factors including work demands, social pressures, lifestyle choices, and certain medical conditions (Bonnet and Arand, 1995; Broman et al., 1996). CSR is associated with deficits in cognitive performance and changes in mood regulation (reviewed in Banks and Dinges, 2011; Jackson et al., 2013). CSR also has negative effects on metabolic, endocrine, and immune functions, and is a risk factor for diabetes, obesity, and cardiovascular disease (reviewed in Van Cauter et al., 2008; Killick et al., 2012).

To study the neurobehavioral consequences of CSR and underlying mechanisms, our laboratory has developed a rat model of CSR ('3/1' model) that takes into account the polyphasic sleep patterns of rodents, as well as circadian regulation of sleep. In this model, alternating periods of 3 h of sleep deprivation (SD; using slowly rotating activity wheels) and 1 h of sleep opportunity (no rotation) are continuously imposed over 4 days (Deurveilher et al., 2012). This protocol reduced total sleep time by ~60% from baseline levels, and initiated both homeostatic and allostatic (adaptive) sleep responses that were strongly modulated by time of day. Homeostatic responses included rebound increases in non-rapid eye movement sleep (NREMS) and rapid eye movement sleep (REMS), and in NREMS electroencephalogram (EEG) delta power, a well-established measure

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Abbreviations: BDNF, brain-derived neurotrophic factor; CSR, chronic sleep restriction; EEG, electroencephalogram; hrBDNF, human recombinant BDNF; LWC, locked wheel control; NREMS, non-rapid eye movement sleep; SD, sleep deprivation; SR, sleep restriction; TBS-T, Tris-buffered saline solution with Tween-20; WRC, wheel-running control.

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of sleep intensity (Borbely and Achermann, 1999), during intermittent sleep opportunities across the 4 days. Allostatic responses included a gradual decline in the rebound of NREMS delta power during sleep opportunities over the 4-day period, and muted rebounds in NREMS and REMS, and the absence of rebound in NREMS delta power, after the 4 days of sleep restriction (SR; Deurveilher et al., 2012). Similar allostatic sleep responses to CSR have been reported by others, also in rodents (Kim et al., 2007, 2012; Clasadonte et al., 2014; but see Leemburg et al., 2010).

The molecular mechanisms underlying the allostatic sleep responses to CSR are largely unknown. One molecule possibly involved in this process is brain-derived neurotrophic factor (BDNF), a member of the nerve growth factor family of neurotrophins with a critical role in neuronal survival and differentiation during development, and in synaptic plasticity in both developing and adult brains (reviewed in Lu, 2003; Park and Poo, 2013). Several lines of evidence implicate BDNF in the regulation of NREMS EEG delta power. Levels of BDNF mRNA in the cerebral cortex increased after 6 h of SD, and this increase was positively correlated with an increase in slow wave (or delta) activity during subsequent sleep in rats (Huber et al., 2007b). Furthermore, cortical BDNF injections increased slow wave activity during NREMS, while injections of a function-blocking BDNF antibody reduced it (Faraguna et al., 2008). In humans, the Val66Met polymorphism in the BDNF gene, which impairs activity-dependent BDNF protein secretion, was associated with reduced NREMS EEG delta power during both baseline and recovery following 40 h of SD (Bachmann et al., 2012). Thus, BDNF is an attractive molecule to study for understanding mechanisms involved in the allostatic EEG delta responses to CSR.

In the present study, we examined whether the 3/1 CSR protocol affects the brain levels of BDNF protein in rats, using Western blot analysis. We evaluated the changes in BDNF levels after either 27 or 99 h of CSR (Experiment 1), and after 24 h of unrestricted sleep following 99 h of CSR (Experiment 2). Wheel-running control (WRC) and locked wheel control (LWC) rats were allowed to sleep *ad libitum*. Levels of mature BDNF were measured in the frontal cortex and basal forebrain, two brain regions known to be involved in regulating sleep and EEG activity including slow waves (reviewed in Deurveilher and Semba, 2011; Riedner et al., 2011), and the hippocampus, an area that is not known to be directly involved in sleep regulation.

EXPERIMENTAL PROCEDURES

Animals

Adult male Wistar rats (n = 42; Charles River Canada, St. Constant, Quebec, Canada) were used; they weighed 296–412 g at the start of the CSR/control protocol. Upon arrival, animals were housed in pairs under a 12-h-light: 12-h-dark cycle (lights on at 07:00 AM) in a temperature-controlled (23 ± 1 °C) animal colony room, with *ad libitum* access to food and water, for a minimum of one week prior to the experiment. Animal-handling procedures followed the guidelines of the Canadian Council on Animal Care, and were approved by the Dalhousie University Committee on Laboratory Animals.

The 3/1 CSR protocol

The 3/1 CSR protocol was conducted by housing rats individually in programmable, motorized activity wheels (11 cm in width, 36 cm in diameter; model 80860, Lafayette Instrument, Lafayette, IN, USA), as previously described (Deurveilher et al., 2012). The wheels were placed inside individual experimental chambers which were each equipped with a fan and a light controlled by a timer to maintain the same 12-h-light: 12-h-dark cycle as in the animal colony room. Food and water were available *ad libitum*. Rats were subject to alternating periods of 3 h of SD imposed by slow rotation of the activity wheel (at ~2.5 m/min), and 1 h of sleep opportunity (no wheel rotation) continuously for either 27 h (Experiment 1) or 99 h (Experiments 1 and 2). Although all the wheels were programed to rotate at the same rate, we found that the actual number of rotations varied slightly among them, presumably due to variability in their mechanical performance (see the Results). When the wheels were rotating slowly during the SD periods, rats adjusted postures, walked slowly, or rode in the wheel for a few seconds. During these 'riding' intervals, rats could eat, drink, groom, or lie down.

Experimental design

Experiment 1. Rats were randomly divided into four treatment groups (n = 6/group): (1) SR2 group, which was sleep restricted for 27 h; (2) SR5 group, which was sleep restricted for 99 h; (3) WRC group; and (4) LWC group.

The SR2 and SR5 rats were housed in locked activity wheels for a 4 or 5-day habituation period prior to the 3/1 CSR protocol. During the habituation period, the activity wheels rotated for periods of 5–20 min (at ~2.5 m/min) once a day during the light phase, to allow the rats to become accustomed to the wheel rotations; the wheels were otherwise locked. The 3/1 CSR protocol began at lights on and continued for either 27 h (i.e., until the end of the first 3 h of SD in the light phase on day 2 of CSR [SR2]) or 99 h (i.e., until the end of the first 3 h of SD in the light phase on day 5 of CSR [SR5]).

The WRC rats were housed in wheels that were unlocked at all times, so rats could turn them freely, for a 9 or 10-day period to match the habituation and CSR periods in the SR5 group. The amount of activity in the WRC rats was monitored as the number of wheel rotations per h using AWM software (Lafayette). The LWC rats were housed in locked activity wheels and left undisturbed, also for a period of 9 or 10 days.

Experiment 2. In Experiment 1, we observed increases in BDNF protein levels in the SR2 and SR5 groups (see Results). To assess whether BDNF protein levels would return to control levels 24 h after 4 days of

the 3/1 protocol, rats were randomly divided into three treatment groups (n = 6/group): (1) SR5 group, which was sleep-restricted for 99 h; (2) recovery (R) group, which was sleep restricted for 99 h and then allowed an opportunity for undisturbed sleep for 24 h (locked wheels); and (3) LWC group, which were housed in locked activity wheels for a time interval matched to that of the R group. The SR5 and R rats underwent a 4 or 5-day habituation period in stationary activity wheels (except for the daily 5–20-min periods during which wheels were slowly rotated to accustom the rats to the wheel rotations) before the start of the 3/1 CSR protocol, as described in Experiment 1.

The body weight of each rat was recorded prior to and immediately following its respective experimental protocol (Experiments 1 and 2).

Tissue collection

At the end of the respective control/CSR protocol (i.e., 10:00 AM), rats were anaesthetized with isoflurane and decapitated. Brains were immediately removed and placed on a cold metal plate to collect frontal cortex, basal forebrain, and hippocampus tissues. The frontal cortex tissue was obtained by isolating the most anterior 5-mm length of the cerebral cortex from both hemispheres. The entire hippocampus (including both dorsal and ventral hippocampi) was removed from both hemispheres. The basal forebrain block was dissected according to Basheer et al. (1999). Briefly, with the ventral surface of the brain facing up, a ~1-mm-thick coronal slice was obtained by cutting in front of and behind the optic chiasm (i.e., at ~0.25 and 1.25 mm from bregma, respectively; Paxinos and Watson, 1998). The slice was then cut horizontally halfway between the anterior commissure and the optic chiasm, to keep the ventral portion. Next, on each side of midline, one vertical cut was made 1 mm lateral to the third ventricle, and a second further laterally through the middle of the olfactory tubercle. These cuts yielded a block of basal forebrain tissue (containing the horizontal diagonal band of Broca region, substantia innominata, and magnocellular preoptic nucleus), measuring approximately 1 mm × 1 mm × 2 mm, from each side.

The tissue samples were immediately placed into separate microcentrifuge tubes that had been buried in dry ice. The tubes containing tissues were then sealed, and transferred to a −80 °C freezer for storage.

In Experiment 2, as an indirect measure of hypothalamic–pituitary–adrenal (HPA) axis activity and chronic stress (Coenen and van Luijtelaar, 1985; Youngblood et al., 1997; Suchecki and Tufik, 2000), adrenal glands were collected from both sides and weighed; the weights were averaged for each rat.

Tissue preparation

Frontal cortex, basal forebrain, and hippocampus tissues were homogenized on ice in NP40 lysis buffer (20 mM Tris, pH 8.0; 137.5 mM NaCl; 10% glycerol; 0.1% NP40; 10 μg/ml leupeptine; 10 μg/ml aprotinin; 1 mM phenylmethylsulfonyl fluoride; 1.5 mM sodium vanadate). Lysis buffer was added to tissue at a ratio of approximately 10:1 (by weight). The homogenates were incubated at 4 °C on a shaker for 20 min and centrifuged at 13,000 rpm for 4 × 40 min at 4 °C (following each cycle of centrifugation, the supernatant from each sample was removed and transferred to a clean microcentrifuge tube for the next centrifugation). Protein concentrations were determined in the final supernatants using a Bio-Rad Protein Assay Kit (catalog #: 500-0002; Hercules, CA, USA). Samples containing 75 μg of protein (frontal cortex and basal forebrain) and 30 μg of protein (hippocampus) were combined with 2× sample buffer and placed in a −20 °C freezer overnight.

Western blotting

Western blotting was carried out following a protocol adapted from Fawcett et al. (1997). Samples were heated at 95 °C for 5 min, centrifuged briefly, and immediately loaded onto 15% polyacrylamide gels. Human recombinant BDNF (hrBDNF; PeproTech, Rocky Hill, NJ, USA), which has been shown to have identical mobility to rat mature BDNF (~14 kDa; Fawcett et al., 1997), was also loaded onto the gels to serve as a positive control. Each gel in Experiment 1 contained two samples from each of the LWC, WRC, SR2, and SR5 groups, while each gel in Experiment 2 contained three samples from each of the LWC, SR5, and R groups. Proteins were separated via electrophoresis at 125 V for 2 h and transferred onto polyvinyl difluoride membranes (Bio-Rad) for 3 h, at 100 V. Membranes were then blocked for 1 h in a blocking solution (5% non-fat milk solution in Tris-buffered saline solution with Tween-20 (TBS-T)) and then further incubated for 72 h at 4 °C in the blocking solution containing a rabbit anti-BDNF antibody (1:200; catalog #: sc-546 (N-20); Santa Cruz Biotechnology, Santa Cruz, CA, USA). This antibody was raised against a peptide mapping within an internal region of BDNF of human origin, and recognized a band corresponding to mature BDNF (14 kDa) in Western blot analysis (manufacturer's technical information; see Fig. 1C, lane 1). Following incubation, membranes were washed for 3 × 10 min in TBS-T and incubated for 1 h at room temperature in TBS-T containing a protein A-horseradish peroxidase-conjugate antibody (1:10,000; catalog #: 170–6522; Bio-Rad). Next, membranes were washed for 3 × 10 min in TBS-T and developed using enhanced chemiluminescence (ECL Plus; Thermo Fisher Scientific, Waltham, MA, USA) for 5 min. Membranes were then scanned using a Typhoon Variable-Mode Imager (Amersham Biosciences/GE Healthcare, Piscataway, NJ, USA).

After scanning, membranes were stripped (ReBlot Plus Strong Antibody Stripping Solution; Millipore, Billerica, MA, USA) and incubated overnight at 4 °C with a mouse antibody against β-tubulin (1:250,000; catalog #: T4026; Sigma Aldrich, St. Louis, MO, USA). Membranes were then washed in TBS-T, incubated in horseradish peroxidase-conjugated secondary antibody, washed again in TBS-T, and visualized as described above, to serve as a loading control.

In addition to the use of hrBDNF as a positive control, the blocking peptide (Catalogue #: sc-546; Santa Cruz) for the anti-BDNF antibody used in the present study

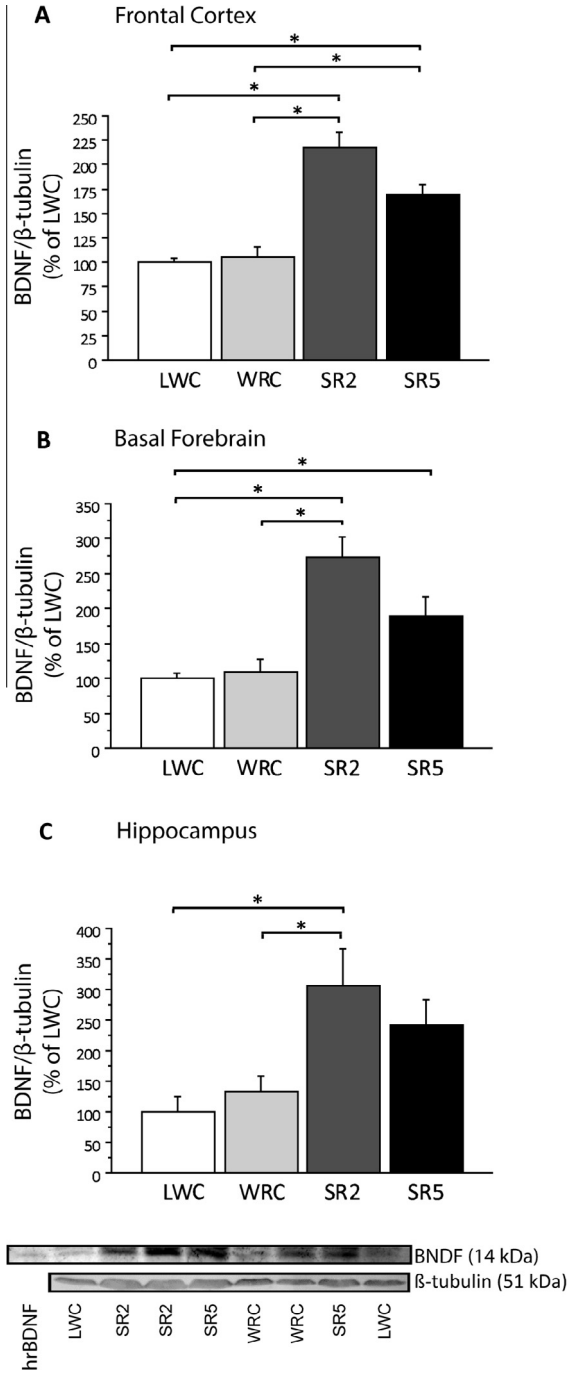

Fig. 1. Effects of 27 and 99 h of sleep restriction on mature brain-derived neurotrophic factor (BDNF) protein levels in the frontal cortex (A), basal forebrain (B), and hippocampus (C). The locked wheel control (LWC) group (white, $n = 6$) was housed in stationary wheels, while the wheel-running control (WRC) group (light gray, $n = 6$) was housed in unlocked wheels that rotated freely, for a time period that matched the SR5 group. The SR2 (dark gray, $n = 6$) and SR5 (black, $n = 6$) groups underwent 27 and 99 h of 3/1 CSR protocol, respectively. The amount of mature BDNF was first normalized to the value for β-tubulin in the same lane on the membrane, and then expressed as a percent of the average level of mature BDNF in the LWC group in the same gel for each animal. A representative western blot from homogenized hippocampus samples (C, bottom) shows bands at 14 kDa, representing mature BDNF, and bands at 51 kDa, representing the loading control β-tubulin in all samples. Human recombinant BDNF (hrBDNF, 14 kDa) was used as a positive control. (*$p < 0.05$, Tukey post hoc tests).

was used for preadsorption control. When the anti-BDNF antibody (1:200) was preincubated with a fivefold excess of the blocking peptide for ~24 h, the band at 14 kDa was completely abolished (data not shown).

Data analyses and statistics

An experimenter (J.W.), blinded to the treatment group, conducted densitometry measurements of Western blot signals using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Each band representing mature BDNF (~14 kDa) was first normalized to the band representing β-tubulin in the same lane. In order to account for possible differences in intensity levels between the scanned membranes, these values were then expressed as a percentage of the average value obtained for the two (Experiment 1) or three (Experiment 2) LWC rats within the same gel. In addition to mature BDNF, we attempted to analyze pro-BDNF (36 kDa). However, the anti-BDNF antibody labeled multiple bands near the molecular weight of pro-BDNF, and we were unable to confidently identify the bands corresponding to pro-BDNF.

Statistical analysis was conducted using Statview 5.0 (SAS Institute, Cary, NC, USA) and SPSS 14.0 (SPSS, Chicago, IL, USA). For each brain region, data were analyzed using a one-way analysis of variance, followed, if applicable, by Tukey post hoc tests. A logarithmic transformation was used to normalize the data and stabilize variances, when required. Probability values < 0.05 were considered statistically significant. All results are shown as means ± standard error of the mean (SEM).

RESULTS

Effects of 27 and 99 h of CSR on BDNF levels (Experiment 1)

The SR2 ($n = 6$) and SR5 ($n = 6$) groups underwent 27 h and 99 h, respectively, of the 3/1 CSR protocol using programed wheel rotations. As controls, the LWC group ($n = 6$) were housed in locked wheels, while the WRC group ($n = 6$) were housed in unlocked wheels that rotated freely, for the same time period as in the SR5 group. The average total number of wheel rotations in the WRC group (13,450 ± 3548 rotations) was not significantly different from the average number of rotations imposed upon the SR5 group (10,021 ± 291) for the same time period (99 h; $t_{10} < 1$, NS). For the SR2 group, 2898 ± 5 rotations were imposed upon the SR2 group over a 27-h period (SR2 < WRC, $t_{10} = 4.06$, $P < 0.005$). As expected for nocturnal rodents, the WRC rats rotated the wheels mostly during the daily dark phase (nocturnal wheel rotations accounted for 95 ± 2% of total daily wheel rotations).

BDNF protein levels. The pattern of BDNF protein levels among the four treatment groups was similar in all three brain regions examined. In the frontal cortex, BDNF levels significantly differed among the four treatment groups ($F_{3,20} = 19.83$, $P < 0.001$; Fig. 1A).

Specifically, the SR2 group had significantly higher BDNF levels than the LWC and WRC groups (218% and 208% of LWC and WRC, respectively; $P < 0.05$ for both comparisons; Fig. 1A). The SR5 group tended to have lower BDNF levels than the SR2 group, but the levels remained significantly higher than in the two control groups (169% and 161% of LWC and WRC, respectively; both $P < 0.05$).

Similarly, BDNF protein levels in the basal forebrain significantly differed among the four treatment groups ($F_{3,20} = 12.99$, $P < 0.001$; Fig. 1B). BDNF levels were significantly higher in the SR2 group than in the two control groups (273% and 251% of LWC and WRC, respectively; both $P < 0.05$), and tended to be attenuated in the SR5 group relative to the SR2 group, although levels remained significantly higher in the SR5 group than in the LWC group (190% of LWC; $P < 0.05$; Fig. 1B).

In the hippocampus (Fig. 1C), BDNF protein levels differed significantly across the four groups ($F_{3,20} = 5.28$, $P < 0.01$). BDNF levels were significantly higher in the SR2 group than in the LWC and WRC groups (305% and 228% of LWC and WRC, respectively; $P < 0.05$ for both comparisons; Fig. 1C), and tended to decline in the SR5 group, which was not significantly different from either control group (241% and 180% of LWC and WRC, respectively). A representative Western blot of hippocampus homogenate samples is shown in Fig. 1C.

Body weights. The four groups differed in the average percent change in body weight from pre-protocol values ($F_{3,20} = 10.74$, $p < 0.001$; Table 1). The LWC group gained weight by an average of 9.6% over the 99 h period. The body weight gain in the WRC group (+5.1%) tended to be smaller than in the LWC group over the same time period. Conversely, CSR resulted in a gradual loss of body weight relative to pre-protocol levels, −3.6% and −5.7% in the SR2 and SR5 groups, respectively (Table 1). These decreases in body weight are consistent with those reported in previous CSR studies in rats (Everson and Szabo, 2009; Barf et al., 2012; Deurveilher et al., 2012).

Effects on BDNF levels of 24 h of recovery following 99 h of CSR (Experiment 2)

In this experiment, the R group ($n = 6$) underwent 99 h of the 3/1 CSR protocol, followed by 24-h recovery in locked wheels. For comparison, the LWC group ($n = 6$) was housed in locked wheels over the same time period (123 h), while the SR5 group ($n = 6$) underwent 99 h of the 3/1 protocol with no recovery as in Experiment 1.

BDNF protein levels. Similar to Experiment 1, the SR5 group showed approximately twofold increases in BDNF levels relative to the LWC group in all the three regions examined. In comparison, the R group showed lower BDNF protein levels in all three brain regions, although there were regional differences. In the frontal cortex (Fig. 2A), BDNF levels tended to be higher in the SR5 group (167% of LWC) than in the other two groups, but there was no significant difference among the three groups ($F_{2,15} = 1.37$, NS).

In the basal forebrain (Fig. 2B), BDNF levels differed significantly among the three groups ($F_{2,14} = 9.84$, $P < 0.005$). The SR5 group had significantly higher BDNF levels than the LWC group (214% of LWC; $P < 0.05$), consistent with the results of Experiment 1. The R group had lower BDNF levels than the SR5 group ($P < 0.05$), with no significant difference between the R and LWC groups.

Similarly, BDNF protein levels in the hippocampus differed across the three groups ($F_{2,13} = 4.67$, $P < 0.05$; Fig. 2C). BDNF levels were significantly higher in the SR5 group than in the LWC group (207% of LWC; $P < 0.05$). The levels in the R group were not statistically different from those in the LWC group, showing a trend of recovery.

Body weights and adrenal gland weights. The three treatment groups differed in the average percent change in body weight from pre-protocol values ($F_{2,15} = 40.45$, $p < 0.0001$; Table 2): the LWC group gained body weight (+12.9%) over the 123-h period, whereas both the SR5 (over the 99-h period) and R (over the 123-h period) groups tended to lose body weight (−1.9% and −0.7%, respectively; Table 2). In contrast to the body weight changes, no difference was observed in adrenal gland weights between the three groups ($F_{2,15} < 1$;

Table 1. Body weights in the LWC, WRC, SR2, and SR5 groups (Experiment 1)

Variables	Treatment groups			
	LWC	WRC	SR2	SR5
Body weight, initial (g)	362.7 ± 9.4	317.3 ± 4.7*	387.0 ± 6.5#	380.3 ± 9.0#
Body weight, final (g)	396.3 ± 4.4	333.3 ± 3.7*	373.0 ± 8.9#	358.3 ± 12.8*,#
Change in body weight (%)	+9.6 ± 2.7	+5.1 ± 1.0	−3.6 ± 1.3*	−5.7 ± 3.1*,#

All values represent means ± SEM ($n = 6$/group). The locked wheel control (LWC) rats were housed in stationary wheels, while the wheel-running control (WRC) rats were in unlocked wheels, which they turned freely throughout (see the Methods for further details). The sleep-restricted SR2 and SR5 rats underwent 27 and 99 h of 3/1 CSR protocol, respectively. The body weight of each rat in the LWC, SR2, and SR5 groups was recorded on the day before the start of the LWC/CSR protocol, and at a time-matched interval for the WRC group (body weight, initial; the smaller body weight in the WRC group compared to the other groups was most likely due to the availability of wheels that they could rotate freely during the habituation period). Body weights were recorded again, 5 days later, immediately following the respective control/CSR protocols (Body weight, final). Percentage of change in body weight was calculated in each rat, and averaged in each group.

* Different from LWC.

\# Different from WRC ($p < 0.05$, Tukey post hoc tests).

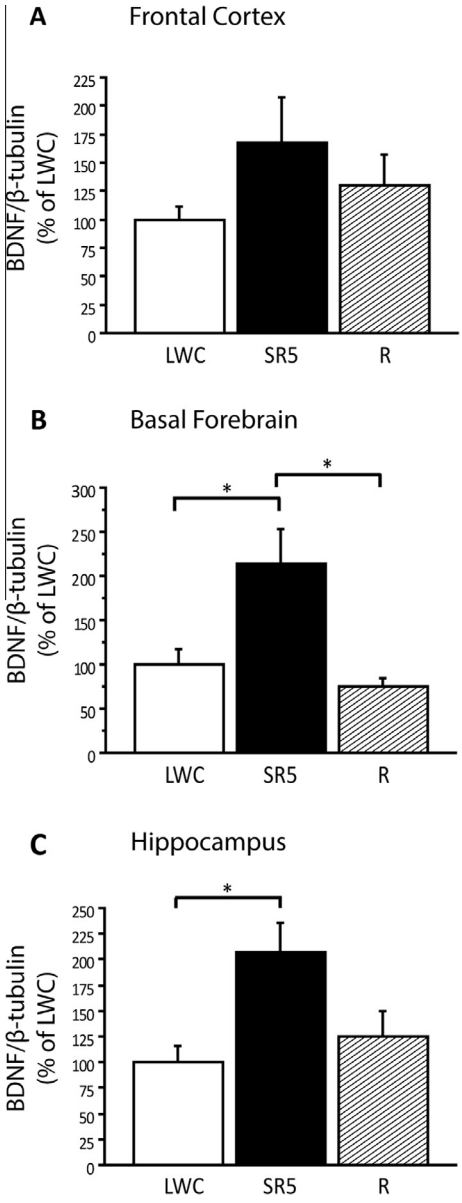

Fig. 2. Effects of 24 h of recovery following sleep restriction on mature brain-derived neurotrophic factor (BDNF) protein levels in the frontal cortex (A), basal forebrain (B), and hippocampus (C). The recovery (R) group (oblique hatches, $n = 6$) underwent 99 h of the 3/1 CSR protocol followed by 24 h of recovery in locked wheels, while the locked wheel control (LWC) group (white, $n = 6$ except $n = 5$ for hippocampus) were housed in locked wheels over the same time period. The SR5 group (black, $n = 5$ except $n = 6$ for frontal cortex) underwent 99 h of the 3/1 CSR protocol. No value was obtained from three samples due to technical difficulties. The amount of mature BDNF was first normalized to the value for β-tubulin, and then expressed as a percentage of the average level of mature BDNF in the LWC group in the same gel for each animal. ($^*p < 0.05$, Tukey post hoc tests).

Table 2; results were similar when adrenal weights were normalized to body weights: $F_{2,15} = 1.41$, NS).

No significant correlations were found between body weight change after 99 h of CSR (SR5) and BDNF levels (normalized to LWC) in any of the three brain regions studied ($r = -0.31$–0.22; all NS; $n = 11$ or 12/ brain region for the SR5 groups in combined Experiments 1 and 2).

DISCUSSION

We found that 27 and 99 h of the 3/1 protocol of CSR elicited two to threefold increases in mature BDNF protein levels in all the three forebrain regions examined, including the frontal cortex, basal forebrain, and hippocampus. The increases after 99 h of CSR were generally smaller than after 27 h of CSR. BDNF levels largely declined to control levels by 24 h after the termination of 99 h of the 3/1 protocol.

CSR-induced increases in BDNF protein levels

Previous studies examining changes in BDNF levels following SD reported rather inconsistent results, most likely due to differences in SR/SD protocols (Cirelli and Tononi, 2000a,b; Taishi et al., 2001; Fujihara et al., 2003; Cirelli et al., 2004; Hairston et al., 2004; Guzman-Marin et al., 2006; Conti et al., 2007; Huber et al., 2007b; Martinowich et al., 2011; Savelyev et al., 2012). Of note, all the previous studies measuring BDNF protein levels used ELISA, which does not allow differentiation of mature BDNF from pro-BDNF, whereas the current study used Western blot analysis, which allows for specific measurement of mature BDNF (in addition to pro-BDNF) protein.

In all three brain regions examined, mature BDNF levels remained above control levels after 99 h of CSR, but the levels were slightly lower than after 27 h. This decline is consistent with a previous study by Cirelli et al. (2006) showing a strong increase in BDNF mRNA levels in the cerebral cortex following 8 h of SD (using gentle handling), and a slightly smaller increase following one week of SD (using the disk-over-water method), although the different methods of SD used at the two time points might have confounded the results in that study.

It is unlikely that increased locomotor activity required by the CSR protocol contributed to the observed increase in BDNF levels. The WRC rats initiated, on average, a similar number of wheel rotations to that imposed on the SR5 rats over the same time period; yet, the WRC rats did not differ from the LWC rats in their BDNF protein levels in any of the three brain regions examined. This lack of effect of voluntary wheel running is consistent with previous reports that BDNF protein (but not mRNA) levels in the hippocampus were unchanged after 7 days of voluntary wheel running (Adlard et al., 2004; Berchtold et al., 2005). These findings, in combination, suggest that any locomotor activity caused by wheel rotations as a means of SD likely did not contribute to the effects of CSR on BDNF levels.

The possibility that the increases in BDNF levels after CSR in the present study was a consequence of stress inherent in the CSR protocol is not supported by reports that acute and chronic stress can decrease BDNF mRNA levels in the hippocampus (e.g., Smith et al., 1995; Kirby et al., 2013) and that corticosterone inhibits BDNF mRNA expression in the hippocampus (e.g., Schaaf et al., 1997, 1998). In addition, acute SD can

Table 2. Body weights and adrenal gland weights in the LWC, SR5, and R groups (Experiment 2)

Variables	Treatment groups		
	LWC	SR5	R
Body weight, initial (g)	362.0 ± 10.4	351.7 ± 4.7	356.3 ± 6.3
Body weight, final (g)	408.3 ± 10.7	345.0 ± 5.2*	353.3 ± 3.2*
Change in body weight (%)	+12.9 ± 0.8	−1.9 ± 1.5*	−0.7 ± 1.4*
Adrenal gland weight (mg)	42.0 ± 3.4	42.2 ± 3.0	42.6 ± 4.0

All values represent means ± SEM (n = 6/group). The LWC rats had locked wheels. The SR5 rats underwent 99 h of the 3/1 CSR protocol, while the recovery (R) rats underwent 99 h of the 3/1 protocol followed by 24 h of recovery in locked wheels. The body weight of each rat was recorded on the day before the start of the control/CSR protocol (body weight, initial). The body weight of each rat in the SR5 group was recorded again 5 days later, following 99 h of the CSR protocol, and the body weight of each rat in the LWC and R groups was recorded again 6 days later, following the respective control/recovery protocols (body weight, final). Percentage of change in body weight was calculated in each rat, and averaged in each group. Adrenal glands were collected and weighed at the end of each experimental protocol.

* Different from LWC ($p < 0.05$, Tukey post hoc tests).

induce BDNF independently of adrenal stress hormones (Mongrain et al., 2010; Thompson et al., 2010). Furthermore, we saw no signs of adrenal hypertrophy in our study, and preliminary results in our laboratory indicate that 99 h of the same CSR protocol did not induce FosB/ΔFosB immunoreactivity (a marker for long-term neuronal activation) in the paraventricular hypothalamic nucleus, which contains neurons that initiate the HPA stress response (Hall et al., 2013). Taken together, these findings suggest that the observed increases in BDNF levels in response to CSR were likely due to sleep loss per se, and not to the stress or increased locomotor activity associated with the CSR procedure.

We also considered the possibility that the difference in BDNF levels between the sleep-restricted and control groups was due to the difference in the immediate sleep/wake history of the animals; i.e., during the first 3 h of the light phase immediately before sacrifice, the CSR animals were kept awake, whereas the LWC and WRC animals likely spent more time asleep as rats usually do at the beginning of the light phase. This possibility, however, is inconsistent with the lower levels of BDNF after 99 vs. 27 h of SR, because the amounts of sleep that occurred during sleep opportunity periods over the first half vs. the second half of the 4 days of the same 3/1 protocol were fairly similar in our previous study using EEG and electromyogram recordings (Deurveilher et al., 2012). In addition, the reported effects of acute (3 h) periods of SD on BDNF levels are discrepant (see above). Furthermore, work from a biosynthesis study of endogenous BNDF in the cortex of rats stimulated with kainic acid showed that maximal levels of proBDNF could be seen by 6 h, while mature BDNF levels peaked 12 h following treatment (Aloyz et al., 1999). Thus it is unlikely that the observed increases in mature BDNF levels were due to the immediate life history of the animals; rather, these increases were more likely a reflection of the chronic condition of SR.

Mechanisms by which the CSR protocol increased BDNF levels are unclear. However, the release of BDNF is strongly regulated by neuronal activity (Aloyz et al., 1999; Matsuda et al., 2009). Thus, increased firing of cortical neurons during SD (Vyazovskiy et al., 2009), as a result of increased release of wake-promoting neurotransmitters during SD (Porkka-Heiskanen et al., 1995; Asikainen et al., 1997; Cirelli and Tononi, 2000a,b; Zant et al., 2011), likely promotes induction of BDNF in the cortex; similar processes might also occur in the other regions.

Recovery of BDNF protein levels following CSR

BDNF levels returned to control levels in all three brain regions studied when rats were allowed 24 h of recovery following 99 h of CSR. As we examined only one recovery time point (24 h), the time course of recovery is unknown. Two previous studies reported that elevated BDNF mRNA levels persisted after 2 h of recovery from 3 or 8 h of SD in the hippocampus, but not in the cortex (Taishi et al., 2001; Hairston et al., 2004). Another study (Fujihara et al., 2003) reported that increased BDNF protein levels in the hippocampus persisted after 3 h of recovery from 6 h of sleep disturbance. The rate of recovery may vary among brain structures, depending on levels of BDNF immediately after CSR, the rates of protein translation and/or degradation, and whether neurotrophin processing enzymes are regulated by these physiological adaptations (Mowla et al., 2001).

Possible role of BDNF in allostatic responses to chronic sleep loss

It is noteworthy that the time course of changes in BDNF protein levels in response to the 3/1 protocol of CSR observed in the present study closely parallels the changes in NREMS EEG delta power (an index of sleep intensity) during the same CSR protocol as reported in our previous study (Deurveilher et al., 2012). In that study, NREMS EEG delta power increased robustly after 1 day of the protocol, but gradually declined over the next 3 days of SR while remaining above baseline levels; after CSR, NREMS EEG delta power was either below baseline levels (during the light phase) or at baseline levels (during the dark phase). Similar observations have been reported (Kim et al., 2007, 2012; Clasadonte et al., 2014), and the lack of compensatory responses in NREMS delta activity has been interpreted as an allostatic response to CSR [allostasis is defined as an adaptive process that serves to maintain an apparent steady state in response to changing external demands (Karatsoreos and McEwen, 2011)]. This parallelism between BDNF levels and NREMS EEG delta power is consistent with the

proposed role of BDNF in the generation of NREMS delta activity (see Introduction), and suggests that BDNF might be part of the mechanisms involved in allostatic adaptation to CSR and play a role in starting off these mechanisms.

Two lines of evidence are relevant for considering a role of BDNF in adaptive cellular, physiological and behavioral processes consistent with allostasis in response to CSR. One is a role of BDNF in synaptic plasticity in the cortex (Lu, 2003). Elevated BDNF protein levels in the cortex have been suggested to promote synaptic potentiation as well as cell-to-cell coupling and, consequently, facilitate neuronal synchrony, leading to an increase in NREMS EEG delta activity (Tononi and Cirelli, 2006; Huber et al., 2007a; Faraguna et al., 2008; Martinowich et al., 2011). The second line of evidence is that BDNF has neuroprotective effects (reviewed in Park and Poo, 2013; Singh and Su, 2013). As SD can induce cellular and oxidative stress in the brain (e.g., Ramanathan et al., 2002; Zhang et al., 2014), elevated levels of BDNF may reflect a neuroprotective response to cellular and physiological stress induced by CSR.

In light of these lines of evidence, it is possible that the fairly sustained increase in BDNF levels during 4 days of CSR will activate molecular factors and cellular processes that serve to help animals to cope with CSR. Once these processes are initiated, sustained increase in BDNF may not be necessary, consistent with the decline in BDNF on the first day of recovery after CSR when various allostatic responses are still observed (see above). The decline may also be beneficial as persistently high levels of BDNF can have negative consequences on brain function (e.g., seizures; Heinrich et al., 2011). Mechanisms that are downstream of BDNF signaling may lead to persistent molecular or genetic alterations that might affect subsequent responses to similar CSR challenge.

Finally, although BDNF may have beneficial consequences on brain function, allowing adaption and protection against CSR, allostatic changes may not be entirely advantageous. We recently found that the 3/1 CSR protocol induced deficits in sustained attention (Deurveilher et al., 2013), and another recent study showed impaired spatial memory in rats sleep-restricted for 5 days (McCoy et al., 2013). The duration and severity of CSR would certainly impact the outcome.

CONCLUSION

Sleep restricting rats for 27 or 99 h induced robust increases in BDNF protein levels in the frontal cortex, basal forebrain, and hippocampus. The time course of these effects paralleled the pattern of changes in NREMS EEG delta power observed in our previous study (Deurveilher et al., 2012), consistent with the role of BDNF in NREMS delta activity. In light of the multiple roles of BDNF in neuronal plasticity and its neurotrophic and neuroprotective properties (Lu, 2003; Park and Poo, 2013; Singh and Su, 2013), the changes in BDNF levels in the frontal cortex, basal forebrain, and hippocampus may play a role in initiating the molecular mechanisms underlying allostatic adaptation to CSR.

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